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Protective effect of a phenolic-rich fraction from *Schisandra chinensis* against H_2O_2-induced apoptosis in SH-SY5Y cells

Chang Hwa Jung, Myung Hee Hong, Ji Hye Kim, Ji Yun Lee, Seong Gyu Ko, Kiho Cho and Ho Moon Seog

Abstract

We have investigated the neuroprotective effects of a phenolic-rich fraction (PRF) on the hydrogen peroxide (H_2O_2)-induced apoptosis of cultured SH-SY5Y cells. The PRF was obtained from the 80% ethanol extract of the fruits of *Schisandra chinensis* by Sepabeads SP-850 column chromatography. Cell viability assays revealed that pretreating SH-SY5Y cells with PRF (10–200 μgmL^{-1}) resulted in significant dose-dependent protection against H_2O_2-induced cell death. The protective effect of PRF against H_2O_2-induced apoptosis was assessed by flow cytometric analysis of DNA contents using propidium iodide (PI) staining. Pre-incubation of cells with PRF at different concentrations for 24 h partially protected apoptosis by H_2O_2 (150 μM). Moreover, cells treated with PRF reduced H_2O_2-induced caspase-3 activation and poly (ADP-ribose) polymerase cleavage and exerted an apparent suppressive effect on oxidative stress induced by reactive oxygen species (ROS). We concluded that PRF may be useful in the treatment and prevention of neurodegenerative diseases associated with elevated ROS levels.

College of Oriental Medicine, Kyunghee University, Seoul 130-701, Republic of Korea

Chang H. Jung, Myung H. Hong, Ji H. Kim, Ji Y. Lee

Institute of Oriental Medicine, Kyunghee University, Seoul 130-701, Republic of Korea

Seong G. Ko

Department of Cardiovascular and Neurologic Disease, Kyunghee University, Seoul 130-701, Republic of Korea

Kiho Cho

Korea Food Research Institute, Kyunggi-do 463-346, Republic of Korea

Ho M. Seog

Correspondence: S. G. Ko, Laboratory of Clinical Biology and Pharmacogenomics, College of Oriental Medicine, Kyunghee University, Republic of Korea. E-mail: epiko@khu.ac.kr

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Introduction

The fruits of *Schisandra chinensis* have been used traditionally in China to treat dyspnoea, cough, mouth dryness, spontaneous diaphoresis, nocturnal diaphoresis, nocturnal emission, dysentery, insomnia and amnesia (Ou 1992; Hancke et al 1999). Chemical investigations of the extracts of *S. chinensis* have revealed the presence of lignans such as schisandrin A, B and C, and gomisin, which are generally believed to be responsible for its reported pharmacological activity (Ikeya et al 1979, 1990; He 1997). These lignans have been reported to have anticancer (Chen et al 2002), anti-hepatocarcinogenic (Nomura et al 1994; Ohtaki et al 1996), anti-hepatotoxic (Wu et al 2003), anti-HIV (Chen et al 1997), antioxidant (Lu & Liu 1992), and anti-inflammatory (Yasukawa et al 1992) activity.

Reactive oxygen species (ROS) have been suggested to be a major cause of neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Simonian & Coyle 1996). Hydrogen peroxide (H_2O_2), a major ROS, exerts its toxic effects mainly through forming the highly reactive hydroxyradical in the presence of the ferrous ion (Fenton 1984). Recent studies have shown a close association between H_2O_2 and neurodegenerative disease, and it has been suggested that H_2O_2 levels are increased during pathological conditions such as ischaemia and neurodegenerative diseases. Hyslop et al (1995) measured H_2O_2 concentration in rat brain using a microdialysis system during ischaemia and reperfusion, and found that a significant rise in H_2O_2 occurred after approximately 1-h reperfusion. β-Amyloid, which is a known cause of Alzheimer's disease, also increased H_2O_2 levels (Behl et al 1994). Moreover, because H_2O_2 readily traverses membranes it may exert cytotoxic effects on cells in the proximity of those responsible for its production (Halliwell 1992).

Apoptosis is triggered under a variety of physiological conditions and is tightly regulated by a number of gene products that promote or block cell death at different stages. H_2O_2-induced apoptosis was observed in a neuronal cell line (Whittemore et al 1994; Satoh et al

1996) and the prevention of H_2O_2-induced neuronal apoptosis may be a useful strategy for the prevention and treatment of neurodegenerative diseases. Moreover, apoptosis prevention was recently suggested as a novel means of neuroprotection (Guo et al 2005). In this study, we have investigated whether a fraction of the ethanolic extract of *S. chinensis* was able to protect human neuroblastoma SH-SY5Y cells from H_2O_2-induced apoptosis.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco Life Technologies (MD, USA). Poly (ADP-ribose) polymerase (PARP) monoclonal antibody was purchased from BD Biosciences (CA, USA), and cleaved caspase-3 monoclonal antibody from Cell Signaling Technology Inc (MA, USA). PD98059, SB203580 and SP600125 were purchased from Calbiochem (CA, USA), and 2′,7′-dichlorodihydrofluorescein (DCHF-DA) was purchased from Molecular Probes Inc. (OR, USA). Electrochemiluminescence detection agents were from Amersham Biosciences (Piscataway, NJ), and phenyl-methylsulfonylfluoride (PMSF), 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), lipopolysaccharide (LPS), and all other chemicals were from Sigma-Aldrich Co. (MO, USA).

Sample preparation

The dried fruits of *S. chinensis* were purchased from Omniherb (Yeongcheon, Korea). A voucher herbarium specimen of *S. chinensis* was deposited in the herbarium of the College of Oriental Medicine, Kyunghee University, Seoul. *S. chinensis* (100 g) was extracted twice by repeat sonication (30 min with 80% ethanol) and then filtered. Filtrates were combined and concentrated using a vacuum evaporator, and the concentrate obtained was lyophilized. The yield of lyophilized extract was 41.5%. The lyophilized extract was then re-dissolved in 80% ethanol and run through a column (2×30 cm) of Sepabeads SP-850 resins (Mitsubishi, Tokyo, Japan). These are cross-linked polystyrene resins for the adsorption of aromatic-type compounds (e.g. phenolic compounds). The crude phenolic fraction was obtained as an 80% ethanolic eluent (200 mL) after washing the column with distilled water (300 mL) to remove water soluble components. The fraction was concentrated in a vacuum evaporator and re-dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 mg mL^{-1}.

Determination of total phenolic level and of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the phenolic-rich fraction (PRF)

Total phenolic contents in the PRF were determined using Folin-Ciocalteu's reagent according to the method described by Singleton et al (1999), with some modifications. Each concentration of PRF (1 mL) and 1 mL 50% Folin-Ciocalteu reagent were mixed, and 3 min later 1 mL 10% sodium carbonate was added. After leaving the reaction for 1 h, the concentration of total phenolic contents was measured by reading the absorbance at 760 nm. Gallic acid was used as standard. The antioxidant activity of PRF was assessed on the basis of the radical scavenging effect of the stable DPPH free radical. A 20-μL sample of extract was added to 0.2 mL DPPH (0.1 M) in methanol solution in a 96-well microplate. After incubation at 37°C for 30 min, the absorbance of each solution was determined at 540 nm using the ELISA microplate reader (Model 550; Bio Rad Laboratories Inc., CA). The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [1-(\text{Absorbance}_{\text{sample}}/\text{Absorbance}_{\text{control}})]\times 100$$

Cell culture

Human neuroblastoma SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in DMEM medium supplemented with 2 mM glutamine, antibiotics (100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin) and 10% (v/v) heat-inactivated fetal bovine serum, and maintained at 37°C in a humidified 5% CO_2 incubator.

Cell viability assays

The MTS test is based on the capacity of viable cells to metabolize (via the mitochondrial succinate dehydrogenase) a yellow tetrazolium salt (MTS) in the presence of phenazine methosulfonate (PMS), acting as an electron coupling agent, to a purple formazan, directly soluble in tissue culture medium and measured by the amount of 490 nm absorbance proportional to the number of living cells. PRF was dissolved in DMSO and then diluted in cell culture medium. The final concentration of DMSO was 0.1%, a concentration that did not demonstrate any effects on the measured parameters in previous control experiments. SH-SY5Y cells were cultured in a 96-well plate at a concentration of 5×10^5 cells/well for 24 h. Culture medium was then aspirated and cells were exposed to a variety of concentrations of PRF (5–100 μg L^{-1}) or H_2O_2 (10–500 μmol L^{-1}) or both for 24 h. Subsequently, 20 μL MTS dye (1 mg mL^{-1}) was added to the cultures and incubated for 2 h at 37°C. The index of cell viability was calculated by measuring the optical density (OD) of colour produced by MTS dye reduction at 490 nm.

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme and is released into culture supernatant when cell membranes are damaged. The percentage of LDH released was expressed as a proportion of the LDH released into the medium compared with the total amount of LDH present in cells treated with 2% Triton X-100. The activity was monitored as the oxidation of NADH at 530 nm by an LDH assay kit (Roche, Mannhein, Germany).

Flow cytometric analysis of apoptotic cells

Human neuroblastoma SH-SY5Y cells were seeded in 10-cm dishes at a density of 2×10^6/dish for 24 h. The cells were

then treated for 24 h with 150 μM of H_2O_2; the IC50 of H_2O_2 was determined by above cell viability assays. Cells were then trypsinized, washed with phosphate-buffered saline (PBS), and fixed overnight in ice-cold EtOH-T (95% ethanol containing 0.5% Tween 20). Cells (2×10^6) were then washed twice with PBS-B (PBS containing 1% BSA), resuspended in 300-μL PBS-B containing 5 μL RNase (10 mg mL^{-1}) and 10 μL propidium iodide (PI) solution. Proportions of apoptotic cells were determined using a BD FACSCalibur with CellQuest software (BD Biosciences).

Immunoblot analysis for PARP and caspase-3

SH-SY5Y cells were treated with PRF (50, 100 or 200 μg mL^{-1}) and H_2O_2 (150 μM) for 24 h, washed with PBS, and lysed with lysis buffer (in mM: 40 Tris-HCl, pH 7.4, 10 EDTA, 120 NaCl, 0.1% Nonidet p-40, 1 DTT, 1 phenylmethylsulfonyl fluoride, 1 NaF, 1 Na_3VO_4, and protease inhibitors) on ice for 20 min. Cell lysates were obtained by centrifugation at 13 000 *g* for 15 min at 4°C, and protein concentrations were determined using Bio-Rad protein assay kits using bovine serum albumin (BSA) as standard. Equal amounts of protein (30 μg) from cell lysates were dissolved in Laemmli's sample buffer and boiled for 5 min. Proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany), which were then blocked in PBS–0.1% Tween 20 (PBST) containing 1% skim milk and 1% BSA for 1 h at room temperature. The membranes were incubated overnight at 4°C with a 1:1000 dilution of monoclonal anti-PARP, cleaved caspase-3, and α-tubulin antibodies. Membranes were washed three times with PBST and further incubated with horseradish peroxidase-conjugated secondary antibodies (dilution 1:1000) for 1 h at room temperature. Membranes were washed three times with PBST and immunobands were visualized using an enhanced chemiluminescence detection system (Amersham-Pharmacia Biotech, Buckinghamshire, UK).

Measurement of intracellular reactive oxygen species

Cells were challenged by exposing them to H_2O_2, and intracellular oxidative stress was determined by using the fluorescent probe DCHF-DA (Oyama et al 1994; Ferretti et al 2003). The DCHF-DA molecule freely crosses cell membranes and after being incorporated into cells is converted into fluorescent 2,7-dichlorofluorescein (DCF) by an oxidative reaction. Thus, DCHF-DA has been used widely to investigate oxidative damage in intact cells as it can be used to determine the intracellular levels of redox-active substances. Briefly, cells (5×10^5 cells/well) were pretreated with PRF at several concentrations (25, 50, 100 or 200 μg mL^{-1}) for 24 h. Subsequently, 150 μM H_2O_2 was added and cells were incubated for 24 h at 37°C. Cells were then incubated with 50 μM DCHF-DA for 30 min at 37°C. Fluorescence was measured using a Fluoroscan Ascent FL (λ_{emit} 526nm; λ_{excit} 490 nm; Type 374, Labsytems, Finland).

Liquid chromatography–mass spectrometry (LC–MS) analysis

An Agilent (Palo Alto, CA) 1100 series quadrupole LC–MS with an atmospheric pressure chemical ionization (APCI) interface was used in the negative and positive ionization mode. Data was collected using Chemstation software version A.09.03. A GEMINI 5-μm C18 110A column (150 mm × 4.60 mm) (Phenomenex, CA) was used with an injection volume of 10 μL for the HPLC separation. The mobile phases consisted of (A) water and (B) methanol at a flow rate of 0.7 mL min^{-1}. Gradient elution was applied as follows: 0–10 min, 10–70% (B); 10–20 min, 750% (B); 20–30 min, 76% (B); 30–40 min, 80% (B). Schisandrin, gomisin A, and gomisin N were used as standards. Pure standards were purchased from Wako (Osaka, Japan).

Statistical analysis

Data are expressed as the mean ± s.e.m. Statistically significant differences among multiple experimental groups were assessed by two-way analysis of variance followed by Tukey's post hoc test where statistical significance was set at $P < 0.05$.

Results and Discussion

Total phenolic contents and DPPH radical scavenging activity

We prepared a phenolic-rich fraction (PRF) from the fruits of *S. chinensis* by Sepabeads SP-850 column chromatography. The total phenolic contents of PRF approximated 11.2 g/100 g by a colorimetric method (Table 1) and the phenolic content of PRF was 10-fold the ethanolic extract. Table 1 shows the DPPH radical scavenging activity of the ethanolic extract and its PRF, which were found to have IC50 values of 85.9 μg mL^{-1} for the PRF and >100 μg mL^{-1} for the ethanolic extract. The DPPH radical scavenging activity of PRF was dose-dependent from 10 to 100 μg mL^{-1} (data not shown). Chiu et al (2002) reported that the radical scavenging activity of *S. chinensis* was mainly due to lignans such as schizandrins and gomisins.

MTS assay

Figure 1 shows cell viability of the human neuroblastoma SH-SY5Y cell line in the presence of PRF. Cell viability in the presence of PRF was assessed by MTS assay after 24-h treatment. The cytotoxicity of PRF was not apparent at

Table 1 Total phenolic contents and DPPH radical scavenging activity of the ethanolic extract and its PRF of *Schisandra chinensis*

Extracts	Total phenolics (g/100 g extract, dry weight)	DPPH radical scavenging activity (IC50, μg mL^{-1})
80% Ethanol extract	1.17	>100
PRF	11.21	85.9 ± 2.3

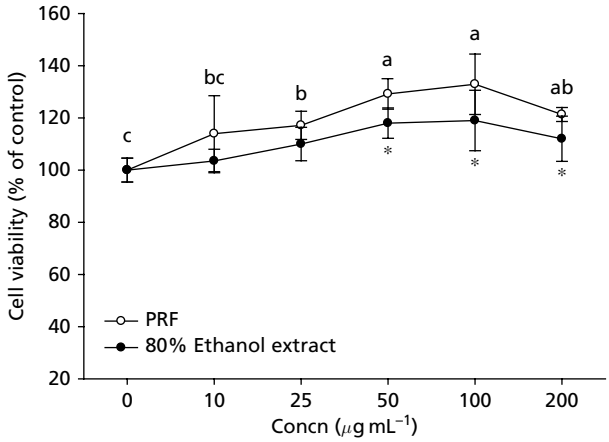

Figure 1 Increased viability of SH-SY5Y cells treated with a PRF (phenolic-rich fraction) obtained from an ethanolic extract of the fruits of *Schisandra chinensis*. Cells (1×10^5 cells/well) were treated with different concentrations of PRF for 24 h and viabilities were determined using the MTS method. Values with non-identical superscripts are significantly different ($P < 0.05$). *$P < 0.05$ vs. 80% ethanol extract within same concentration.

concentrations up to 200 μg mL^{-1}, whereas cell viability appeared to be slightly increased by the PRF (25–100 μg mL^{-1}).

Protective effects of PRF on H_2O_2-treated SH-SY5Y cells

H_2O_2 is a common oxidant, and has been shown to induce apoptosis in several cell types, including PC12 cells and fibroblasts (Makino et al 1994; Liu et al 2005). To study the protective effect of PRF under pathological conditions, we investigated its effect on H_2O_2-induced oxidative stress. Cell viability under oxidative stress was measured using the MTS and LDH leakage assay. LDH leakage and MTS assay gave similar results for effects on cell viability in response to H_2O_2 at both studied concentrations (Figure 2A and B). Our study showed that the viability of SH-SY5Y cells exposed to 150 μM H_2O_2 for 24 h was approximately 50% that of controls, and we used these conditions to treat SH-SY5Y cells.

To determine whether PRF could prevent the cytotoxicity induced by H_2O_2 in SH-SY5Y cells, we examined the effects of PRF on H_2O_2-induced cell death. As shown in Figure 2C, on

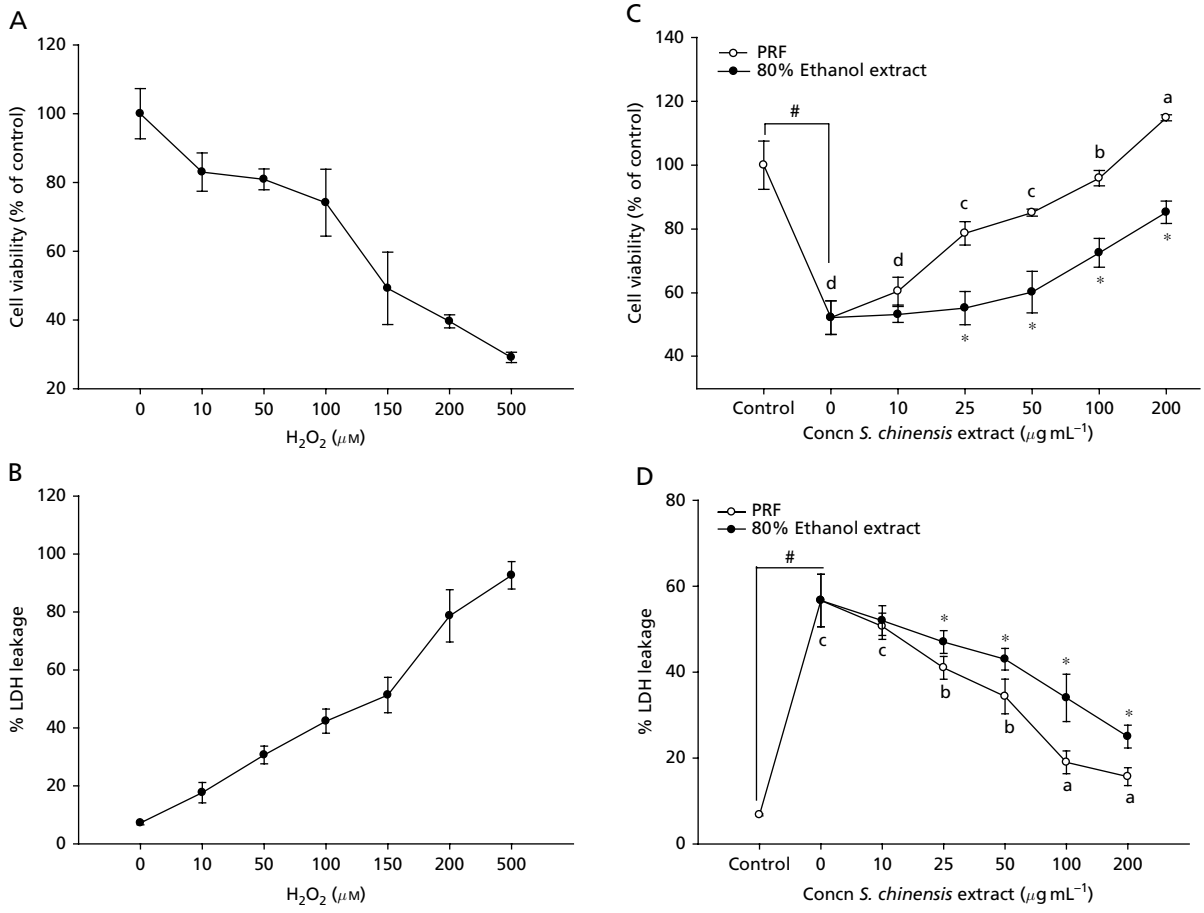

Figure 2 Protective effect of PRF (phenolic-rich fraction) from *Schisandra chinensis* on H_2O_2-induced cytotoxicity in SH-SY5Y cells. Confluent cells in 96-well plates were exposed to various concentrations of H_2O_2 for 24 h and subjected to MTS (A) and LDH leakage (B) assays. Cells were pretreated for 2 h with different concentrations of PRF, and then H_2O_2 (150 μM) was added and cells were further incubated for 24 h and subjected to MTS (C) and LDH leakage (D) assays. Control group: without H_2O_2. Values with non-identical superscripts are significantly different ($P < 0.05$). #$P < 0.05$ compared with control group. *$P < 0.05$ vs. 80% ethanol extract, within same concentration.

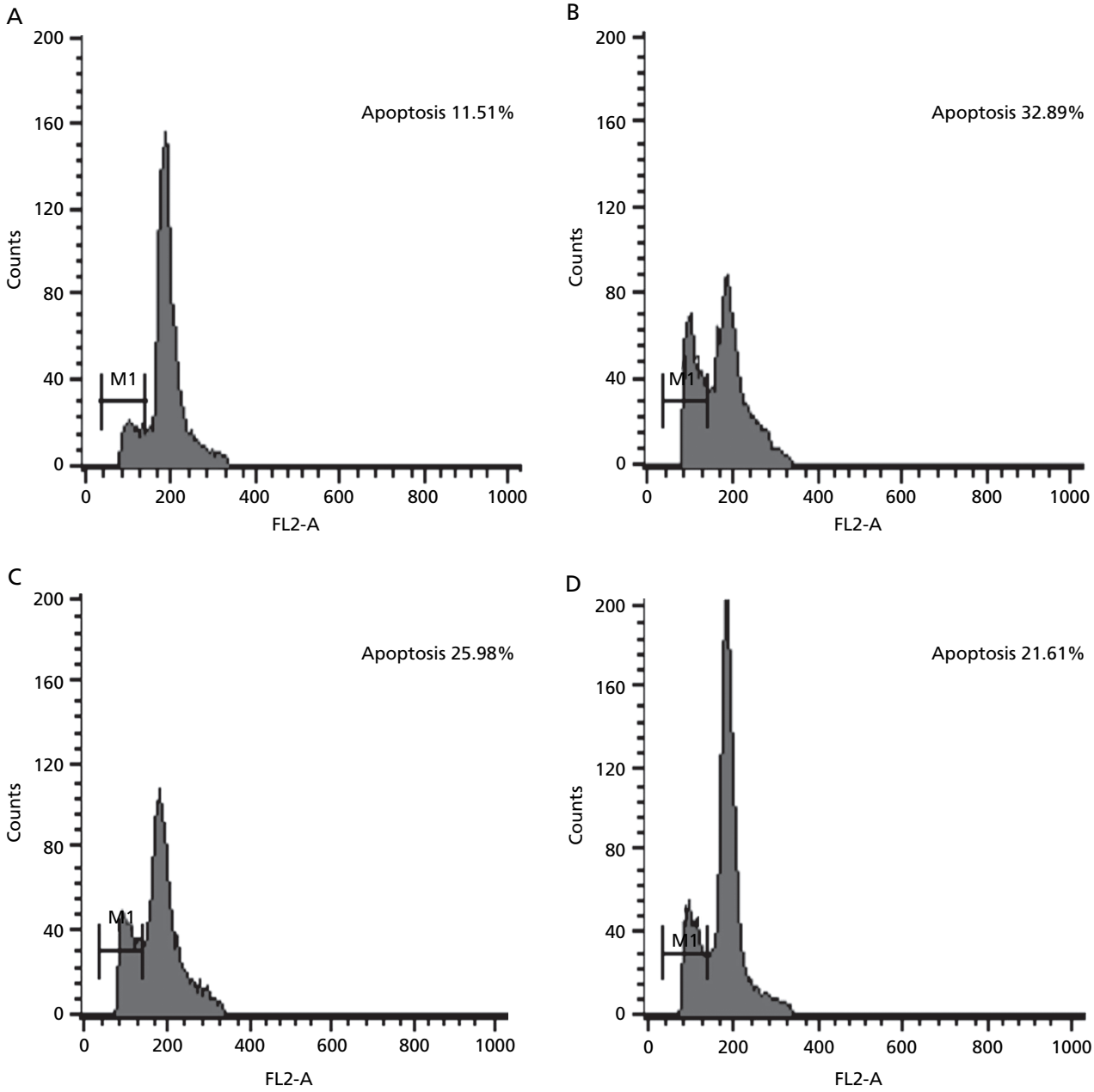

Figure 3 Protective effect of PRF (phenolic-rich fraction) on H_2O_2-induced apoptosis in SH-SY5Y cells by flow cytometric DNA analysis. SH-SY5Y cells (1.5×10^6 cells/dish) were treated with 150 μM H_2O_2 in the presence of PRF for 24 h at 37°C. A, control cells; B, cells treated with H_2O_2 alone; C, cells were treated with H_2O_2 and PRF (50 $\mu g\,mL^{-1}$); D, cells were treated with H_2O_2 and PRF (200 $\mu g\,mL^{-1}$).

increasing PRF concentration from 10 to 200 $\mu g\,mL^{-1}$ for an exposure time of 24 h, its protective effect against H_2O_2-induced cytotoxicity was found to increase in a dose-dependent manner. An improvement in the viability of 115% vs the control group at 200 $\mu g\,mL^{-1}$ for 24 h was shown. Tetrazolium salt reduction may be inhibited without affecting cell viability when an antioxidant compound is tested (Trevisi et al 2006). To confirm our results we used another assay, the LDH leakage method assay, and LDH leakage gave similar results for the effects on cell viability in response to PRF (Figure 2D). Thus, our results indicated that PRF dose-dependently protected SH-SY5Y cells against the cytotoxicity induced by H_2O_2. Several research groups are currently searching for natural substances with neuroprotective effects, and attention has been focused on a wide array of dietary antioxidants that can scavenge free radicals and protect cells from oxidative damage (Altiok et al 2006; Lu et al 2006).

PRF protected SH-SY5Y cells against H_2O_2-induced apoptosis

To determine whether H_2O_2 treatment induced SH-SY5Y cell death via apoptosis, we measured percentages of apoptotic

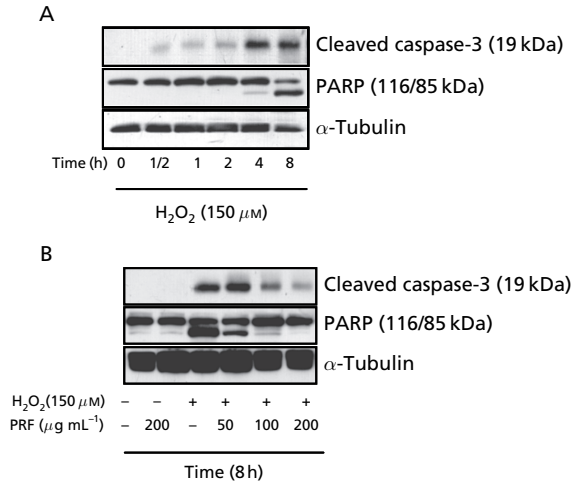

Figure 4 Inhibitory effect of PRF (phenolic-rich fraction) on H_2O_2-induced caspase-3 and PARP cleavage in SH-SY5Y cells. A, SH-SY5Y cells (1.5×10^6 cells/dish) were treated with 150 μM H_2O_2 for various times. B, Cells were treated with 150 μM H_2O_2 for the indicated times in the presence of PRF. α-Tubulin was used as a loading control.

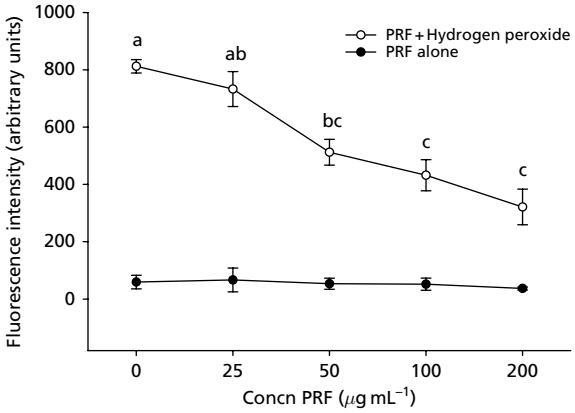

Figure 5 Effect of PRF (phenolic-rich fraction) from *Schisandra chinensis* on H_2O_2-induced intracellular ROS levels. SH-SY5Y cells (1×10^5 cells/well) were treated with 150 μM H_2O_2 for the indicated times in the presence of PRF. Values with non-identical superscripts are significantly different ($P < 0.05$).

cells using a flow cytometer. In flow cytometric histograms, apoptotic cells will give DNA fluorescence in the subdiploid regions. Ten thousand cells were analysed per sample and percentage apoptotic cells in sub-G1 peaks (an indicator of apoptosis) were calculated. The apoptotic cells in the sub-G1 phase in the 150 μM H_2O_2-treated group (Figure 3B) was 32.89%, and PRF treatment decreased this value to 21.61% (Figure 3D). These concurred with MTT assay findings.

PRF inhibited H_2O_2-induced apoptotic protein expression

Caspase-3 has been shown to be an important regulator of apoptosis (Green & Reed 1998; Kang et al 2004), and so we examined the effect of PRF on H_2O_2-induced caspase-3 and PARP activation in SH-SY5Y cells. As shown in Figure 4A, incubation of SH-SY5Y cells with H_2O_2 (150 μM for 8 h) increased cleaved caspase-3 activity. Moreover, pre-incubation with various concentrations of purified PRF for 8 h inhibited H_2O_2-induced caspase-3 activation in a dose-dependent manner. On the other hand, PARP is a 116-kDa nuclear protein that is specifically cleaved by active caspase-3 into an 85-kDa apoptotic fragment. In this study, we also found PRF inhibited PARP cleavage by H_2O_2 (Figure 4B).

PRF reduced H_2O_2-induced ROS production in SH-SY5Y cells

Cells are probably killed by H_2O_2 due to oxidative stress, since H_2O_2 increases intracellular ROS levels. Thus, we investigated the inhibitory effect of PRF on ROS production in the presence of H_2O_2. DCHF-DA reveals the intracellular production of redox-active substances and has been used widely to investigate oxidative damage in intact cells. The nature of the reaction between PRF and ROS in H_2O_2-treated cells is shown in Figure 5. Increased DCF fluorescence intensity caused by H_2O_2 was significantly reduced by PRF. One possible explanation for the effect of PRF on the oxidative stress induced by H_2O_2 concerns its polyphenolic content, because it is known that plant-derived polyphenolics are potent antioxidants and free radical scavengers (Ishige et al 2001), although it is also possible that H_2O_2 is directly scavenged by PRF.

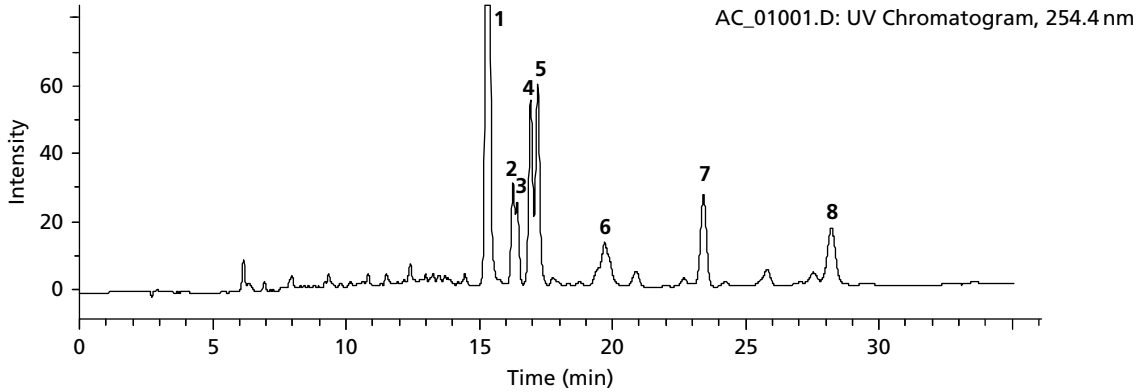

Figure 6 HPLC-MS chromatograms of a phenolic-rich fraction from *Schisandra chinensis*. Peak assignments are shown in Table 2.

Table 2 Peak assignments for analysis for PRF of *Schisandra chinensis*

Peak number	Retention time (min)	$[M+H]^+$	$[M-H]^+$	Identification
1	15.1	–	432	Schisandrin
2	16.2	389	387	Nd
3	16.7	483	–	Nd
4	17.0	417	–	Gomisin A
5	17.4	391	389	Nd
6	19.4	403	401	Nd
7	23.2	417	415	Nd
8	27.8	401	399	Gomisin N

nd, Unable to identify. –, No dominant molecular ion.

Liquid chromatography–mass spectrometry analysis

LC-MS chromatograms of PRF of *S. chinensis* are shown in Figure 6. The value of retention time, $[M+H]^+$, $[M-H]^+$ and the identification of individual peaks are listed in Table 2. Peaks 1, 4 and 8 were identified as schisandrin (1), gomisin A (4) and gomisin N (8), based on their retention time, $[M+H]^+$ and $[M-H]^+$ ions compared with the data of reference compounds. Lignan compounds (schisandrin and gomisin) are known to have various biological activities, especially antioxidant and hepatoprotective effects (Nomura et al 1994; Ohtaki et al 1996; Yoshikawa et al 2006). Recently these compounds have been investigated as to their effects in improving memory (Kim et al 2006; Luo & Liu 2006).

Conclusion

Our data suggested that PRF, obtained from the 80% ethanol extract of the fruits of *Schisandra chinensis*, protected neurons against H_2O_2-induced cell death. Thus, PRF contains principals that may be useful for the prevention and treatment of neurodegenerative diseases associated with ROS.

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